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Constitutive cytoplasmic localization of p21$^{Waf1/Cip1}$ affects the apoptotic process in monocytic leukaemia

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In the present study, we analysed the expression and localization of p21$^{Waf1/Cip1}$ in normal and malignant haematopoietic cells. We demonstrate that in normal monocytic cells, protein kinase C (PKC)-induced p21 gene activation, which is nuclear factor-κB (NF-κB) independent, results in predominantly cytoplasmic localized p21 protein. In acute monocytic leukaemia (M4, M5), monocytic blasts ($N=12$) show constitutive cytoplasmic p21 expression in 75% of the cases, while in myeloid leukaemic blasts ($N=10$), low nuclear and cytoplasmic localization of p21 could be detected, which is also PKC dependent. Constitutive p21 expression in monocytic leukaemia might have important antiapoptotic functions. This is supported by the finding that in U937 cells overexpressing p21, VP16-induced apoptosis is significantly reduced (20.0 ± 0.9 *vs* $55.8\pm3.8\%$, $P<0.01$, $N=5$), reflected by a reduced phosphorylation of p38 and JNK. Similarly, AML blasts with high cytoplasmic p21 were less sensitive to VP16-induced apoptosis as compared to AML cases with low or undetectable p21 expression (42.25 *vs* 12.3%, $P<0.01$). Moreover, complex formation between p21 and ASK1 could be demonstrated in AML cells, by means of coimmunoprecipitation. In summary, these results indicate that p21 has an antiapoptotic role in monocytic leukaemia, and that p21 expression is regulated in a PKC-dependent and NF-κB-independent manner.

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Introduction

Acute myeloid leukaemia (AML) is characterized by an accumulation of immature cells in the bone marrow, resulting in the disruption of normal haematopoiesis.[1–3] The growth advantage of the leukaemic population is in part linked to the constitutive activation of intracellular proteins that trigger the function of antiapoptotic proteins.[4,5] Recent studies have indicated that nuclear factor-κB (NF-κB) is constitutively expressed in AML cells, which is due to the activation of the Ras/PI3 kinase pathway.[4,6–8] A recent study in normal monocytes has suggested that the antiapoptotic function of NF-κB is in part mediated by the cytoplasmic p21$^{Waf1/Cip1}$ (p21) protein.[9,10] So far, p21, a member of the family of cyclin-dependent kinase (CDK) inhibitors, was defined as a nuclear protein. These proteins function by inhibiting CDK1, -2, -4 and -6, and induce cell cycle arrest at the G1/S boundary. This process allows the cells to exit the cell cycle and differentiate.[11–16] Localization of p21 in the cytoplasm has been connected to monocytic differentiation, and is implicated in antiapoptotic functions.[9,10,17] Cytoplasmic p21 can form a complex with apoptosis signal-regulating kinase 1 (ASK1), preventing the activation of the mitogen-activated protein (MAP) kinase cascade.[10] In addition, p21 can bind to procaspase 3, preventing the induction of apoptosis in HepG2 cells.[18,19] Finally, p21 has been shown to be a downstream target of protein kinase B (PKB (AKT)). Two sites in the carboxyl terminus of p21 are phosphorylated by PKB, which enhances the stability of the p21 protein and modulates binding of p21 to target proteins.[20,21]

In view of the link between p21 and apoptosis, we questioned whether p21 expression and localization is perturbed in malignant haematopoietic cells and whether cytoplasmic localization of p21 protects against the cytotoxic effects of chemotherapy. The results demonstrate that p21 is not expressed in unstimulated haematopoietic cells of monocytic and myeloid origin. In addition, it is demonstrated that PMA is capable of inducing p21 expression in a protein kinase C (PKC)-dependent manner. In monocytic leukaemia, p21 is constitutively expressed and cytoplasmic localized, in contrast to myeloid leukaemia where no cytoplasmic p21 was detected. We postulate that cytoplasmic p21 protects these cells against the effects of cytotoxic agents, as is reflected by a decrease in VP16-induced apoptosis in p21-overexpressing cells and AML blasts with constitutive p21 expression.

Materials and methods

Patient population and isolation of AML cells

Peripheral blood cells or bone marrow cells from 22 adult untreated patients with AML were studied after informed consent. The AML cases were defined according to the classification of the French–American–British (FAB) committee as M0–M6.[22] AML blasts were isolated by density-gradient centrifugation. The cells were cryopreserved in aliquots of $20–30\times10^6$ cells in RPMI 1640 (Biowhittaker, Verviers, Belgium) supplemented with 10% dimethylsulphoxide (DMSO; Sigma, St Louis, MO, USA) and 10% foetal bovine serum (FBS; Bodinco, Alkmaar, The Netherlands), employing a method of controlled freezing and storage in liquid nitrogen. After thawing, T-lymphocytes were depleted by 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cell (SRBC) rosetting. The cell population consisted of more than 98% AML blasts, as determined by May–Grünwald–Giemsa staining. Fluorescence-activated cell sorting (FACS) analysis demonstrated <1% CD3 (Becton Dickinson, Sunnyvale, California, USA)-positive cells.

Preparation of monocytes and granulocytes

Peripheral blood cells were obtained from healthy volunteer blood donors, and mononuclear cell suspensions were prepared by Ficoll–Hypaque density-gradient centrifugation. T-lymphocytes were depleted by AET-treated SRBC rosetting. Monocytes

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were further enriched by plastic adherence (1 h, 37°C, 5% CO_2) and demonstrated a purity >95%, detected by FACS analysis with anti-CD14 antibody (Becton Dickinson, Sunnyvale, CA, USA).

Peripheral blood from healthy volunteers, anticoagulated with 0.32% sodium citrate, was used to isolate granulocytes, as described by Koenderman *et al*.[23] In short, mononuclear cells were removed by centrifugation over Fycoll-Paque (Amersham, Upsala, Sweden), and erythrocytes were lysed with ice-cold NH_4Cl solution. Granulocytes were allowed to recover for 30 min at 37°C in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 0.5% human serum albumin (HSA; CLB, Amsterdam, The Netherlands). Before stimulation, cells were resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$, 0.5% HSA). In all cases, the cell population isolated consisted of >95% granulocytes, as determined by May–Grünwald–Giemsa staining.

Cell culture

AML blasts were cultured at 37°C, 5% CO_2, at a density of 1×10^6/ml in RPMI 1640 media supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (ICN, Costa Mesa, CA, USA) and 10% FBS. Monocytes were cultured at 37°C, 5% CO_2, at a density of 1×10^6/ml in RPMI 1640 and 10% FBS. The human growth factor-dependent cell lines AS-E2[24] and GF-D8[25] were cultured in RPMI 1640 supplemented with 5% FBS and 10 ng/ml IL-3 (Genetics Institute Cambridge, MA, USA). The human cell lines U937 (ATCC, Product No. CRL-1593.2), THP-1 (ATCC, Product No. TIB-202) and HL-60 (ATCC, Product No. CCL-240) were cultured in RPMI 1640 supplemented with 10% FBS. The primitive human cell line KG1a (ATCC, Product No. CCL-2461) was cultured in IMDM (ICN) with 20% FBS. In addition, U937 cells stable transfected with a $ZnCl_2$ (180 μM, 3 days)-inducible p21 construct were used in conjunction with a mock-transfected cell line for studying the cellular function of p21.[10,17]

Reagents and antibodies

Antibodies against phosphorylated extracellular-signal-regulated kinase (ERK), PKB, p38 and c-jun N-terminal kinase (JNK) were obtained from New England Biolabs (Beverly, MA, USA). Antibodies against p38, JNK, ASK1 and an antibody–agarose conjugate for immunoprecipitation of p21 (C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). p21 antibody was purchased from Transduction Laboratories (Lexington, KY, USA), and an antibody against actin was obtained from Boehringer Mannheim (Mannheim, Germany). Supershift antibodies for p50 and p65 were purchased from Santa Cruz Biotechnology, as well as the Rb antibody. Horseradish peroxidase (HRP)-labelled secondary antibodies were obtained from DAKO (Glostrup, Denmark).

Recombinant human (Rh) interleukin (IL)-1β was obtained from Mekesson HBOC Bioservices (Rockville, MD, USA). Rh IL-3 and granulocyte–monocyte-colony-stimulating factor (GM-CSF) were purchased from Genetics Institute (Cambridge, MA, USA), and phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (St Louis, MO, USA) as well as actinomycin D. The p38 MAPK inhibitor SB203580 and the NF-κB inhibitor SN-50 were obtained from Biomol (Plymouth Meeting, PA, USA). The PI3K inhibitor Ly294002 was obtained from Alexis (San Diego, CA, USA). MAP kinase/ERK kinase (MEK) 1 inhibitor U0126 was obtained from Promega Corp. (Leiden, The Netherlands). The PKC inhibitors bisindolylmaleimide I (BIM or GF 109203X), chelerythrin and calphostin-C were obtained from Calbiochem (Darmstadt, Germany). VP16 was purchased from TEVA Pharming (Mijdrecht, The Netherlands). Radionucleotides and the random hexamer primers p(CAN)6 were obtained from Amersham (Buckinghamshire, UK). H_2O_2 was obtained from Merck (Darmstadt, Germany). M-MLV-RT polymerase and RNAse inhibitor were obtained from MBI Fermentas (St Leon-Rot, Germany). Tri Reagent was purchased from MRC Molecular Research Center Inc. (Cincinnati, OH, USA). The Quantitect SYBR Green PCR Kit was obtained from Qiagen GmbH (Hilden, Germany). The dNTPs were purchased from Invitrogen BV (Breda, The Netherlands).

Combined annexin V/propidium iodide staining procedure

Viability was assessed using an annexin V staining kit (IQ Products, Groningen, The Netherlands), according to the manufacturer's recommendations. Briefly, after 24 h of culture in RPMI 1640 medium supplemented with 10% FBS with or without addition of VP16 (20 μg/ml), cells were harvested, resuspended in 100 μl calcium buffer containing 5 μl of annexin V and incubated for 20 min at 4°C in the dark. Cells were washed with 5 ml calcium buffer, and subsequently incubated in 300 μl calcium buffer containing 2.5 μl of propidium iodide (PI) for 10 min in the dark. Finally, binding of fluorescein-conjugated annexin V and PI was measured by fluorescence-activated cell sorting (Becton Dickinson, Sunnyvale, CA, USA). Apoptosis was also analysed by measuring active caspase 3 levels by flow cytometry, using an FITC-labelled antibody (BD Biosciences, Alphen aan den Rijn, The Netherlands) against the active form of caspase 3. The cells were fixed and permeabilized with fix and perm obtained from BD Biosciences.

Preparation of protein extracts and Western blotting

The amounts of p21, ASK1, p38, JNK and actin, and the degree of phosphorylated p38 and JNK were determined by Western blotting on whole-cell extracts, cytoplasmic or nuclear extracts. U937 cell lines were cultured for 3 days in RPMI 1640 supplemented with 10% FBS, with or without $ZnCl_2$ (180 μM), and overnight with VP16 (20 μg/ml) or H_2O_2 (300 μM). Cells were harvested and total cell extracts were prepared by resuspending the cells in sample buffer (2% SDS, 10% glycerol, 2% β-mercaptoethanol, 60 mM Tris-HCl pH 6.8 and bromophenol blue). Cell extracts were directly boiled for 5 min, and proteins were fractionated by running on SDS-PAGE gel. The proteins were electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA, USA) blocked with TBS buffer containing 0.1% Tween-20 and 5% nonfat milk, prior to incubation with antibodies, diluted in 5% bovine serum albumin. Binding of each antibody was detected by HRP-labelled secondary antibodies, using enhanced chemiluminesence (ECL), according to the manufacturer's recommendations (Amersham Life Sciences, Buckinghamshire, UK).

Monocytes and AML blasts were cultured for 24 h in RPMI 1640 supplemented with 10% FBS with or without PMA (50 nM), IL-1β (10 ng/ml), IL-3 (10 ng/ml), GM-CSF (10 ng/ml), Ly294002 (5 μM), SB203580 (1 μM), U0126 (5 μM), BIM (1 μM), chelerythrin (6 μM) or calphostin-C (100 nM), at a density of 1×10^6 cells/ml. Cells were harvested, and cytoplasmic and nuclear extracts

were prepared according to the 'mini-extracts' method.[26] The extracts were normalized for protein content prior to SDS-PAGE. Proper fractionation and lack of leakage of nuclear proteins to the cytosol were determined by Western blotting against the nuclear protein retinoblastoma (Rb).

To investigate the role of an autocrine growth factor, AML blasts were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, and subsequently stimulated for 6 h with conditioned AML medium (prepared by incubating AML cells at a density of 3×10^6 cells/ml for 3 days) or 50 nM PMA as a control.

RNA extraction, preparation of cDNA and real-time PCR

Monocytes (5×10^6) were cultured for 24 h in RPMI 1640 supplemented with 10% FCS, and subsequently stimulated for 0, 1, 2 or 3 h with 50 nM PMA, in the presence and absence of 1 μg/ml actinomycin D. Cells were lysed in 1 ml Tri reagent, and RNA was isolated according to the manufacturer's recommendations. Briefly, 0.2 ml chloroform was added to the Tri reagent cell mixture, and after 5 min at room temperature the RNA was phase separated into the aqueous phase by centrifuging at 12 000 *g* for 15 min at 4°C. The RNA was precipitated from the aqueous phase by mixing and centrifuging with 1 volume of isopropanol for 10 min at 12 000 *g* at 4°C. After washing the RNA pellet with 1 ml 75% ethanol, it was dried and dissolved in DepC-treated H_2O.

The isolated RNA was used to make cDNA. Briefly, annealing of the random hexamer primers (p(CNA)6) was performed by incubating 5 μl of RNA for 10 min at 65°C in the presence of the random hexamer primers, and immediately putting them on ice afterwards.

The synthesis of the cDNA was then completed by incubating the samples at 37°C for 1 h in the presence of 0.5 mM dNTPs, M-MLV-RT polymerase and RNAse inhibitor.

A real-time PCR analysis of p21 expression was performed using the Quantitect SYBR Green PCR Kit from Qiagen, following the manufacturer's instructions on the Light Cycler system from Roche. The p21 values were normalized against values obtained by real-time PCR for HPRT. The primers used were: p21, FOR; tcaccgagacaccactggag, REV cttccaggactg caggcttc; HPRT, FOR; tggcgtcgtgattagtgatg, and REV gatgtaatc cagcaggtcag.

Electrophoretic mobility shift assay

Monocytes were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, with or without the addition of SN-50 (100 μg/ml), and subsequently stimulated for 6 h with 50 nM PMA. Nuclear extracts were prepared according to the 'mini-extracts' procedure as previously described, divided in small aliquots and stored at –80°C.

Double-stranded synthetic oligonucleotide probes containing the NF-κB (NF-κB: 5′-AGCTGCGGGGATTTTCCCTG-3′) consensus binding sequence were used in the gel retardation assay. The consensus sequence for binding of the nuclear factors is underlined. High-performance liquid chromatography (HPLC)-purified single-stranded oligonucleotide (50 ng) was labelled with T4-polynucleotide kinase and [α^{32}P]dATP (3000 Ci/mmol), separated from nonincorporated radiolabel by sephadex G50 chromatography, ethanol precipitated, dried, and dissolved in 20 μl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT, containing a four-fold excess of the opposite strand. Annealing of the two strands was performed by heating the mixture for 2 min at 90°C, and slow cooling to room temperature. Nuclear extract (5 μg) and 0.1 ng double-stranded labelled oligonucleotide were incubated in 20 mM HEPES (pH 7.9), 60 mM KCl, 0.06 mM EDTA, 0.6 mM DTT, 2 mM spermidine and 10% glycerol, supplemented with 2 μg poly(dI-dC). The binding reaction was performed at 26°C for 25 min. The samples were loaded on prerun (30 min, 150 V) 4% (30:1) polyacrylamide gels, and run for 1 h at 150 V in 0.5 × TBE at room temperature. Gels were dried and exposed to Kodak XAR films at −80°C with an intensifying screen. Quantification of protein binding was performed by densitometry using a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). Competition experiments were performed by adding a 100-fold molar excess of unlabelled double-stranded oligonucleotides. Supershift experiments were performed by incubating the nuclear extracts for 30 min with polyclonal antibodies against the p50 and p65 subunits of NF-κB.

Immunoprecipitation

AML blasts (10^7) were cultured for 16 h in RPMI 1640 medium supplemented with 10% FBS with or without 50 nM PMA. As positive control, 10^7 cells of the human growth factor-dependent cell line AS-E2 were cultured for 16 h in RPMI 1640 supplemented with 5% FBS and 10 ng/ml IL-3. These were cultured in the presence of 50 nM PMA to induce p21 expression. Cells were harvested, washed with ice-cold PBS containing 1 mM sodium orthovanadate, and subsequently lysed in 500 μl lysis buffer (20 mM Tris-HCl pH 7.6, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 10% glycerol, 2 mM Na_3VO_4, 2 mM PMSF, 1 μM pepstatin and 1 mM DTT) for 15 min on ice. Cell lysates were clarified at 10 000 *g* for 20 min, and incubated with 10 μl p21 agarose conjugate (p21 (C-19) AC Santa Cruz, CA, USA) rotating O/N at 4°C. The immune complex was washed three times with lysis buffer and heated in sample buffer, separated by SDS-PAGE, immunoblotted on PVDF membrane (Millipore, Bedford, MA, USA), and incubated with anti-p21 and anti-ASK1 antibodies. Immunocomplexes were detected using ECL (Amersham, UK).

Statistical analysis

The Student's *t*-test for paired samples was used to determine the statistical significance of the apoptosis data in the U937 cell line. The Mann–Whitney *U*-test was used to determine the statistical significance between the two groups of differentially expressing p21 AML blasts.

Results

Expression and regulation of p21 in normal monocytes and myeloid cells

To obtain more information regarding expression, localization and regulation of p21 in normal haematopoietic cells, and especially whether p21 is a downstream target of NF-κB, as is reported earlier,[9] monocytes from healthy donors were stimulated with IL-1β, GM-CSF, IL-3 and the phorbol ester PMA for 4, 6 and 24 h. As depicted in Figure 1a, in unstimulated cells, no p21 protein could be detected, while PMA, IL-3 and GM-CSF all induced p21 expression, with a predominant cytoplasmic

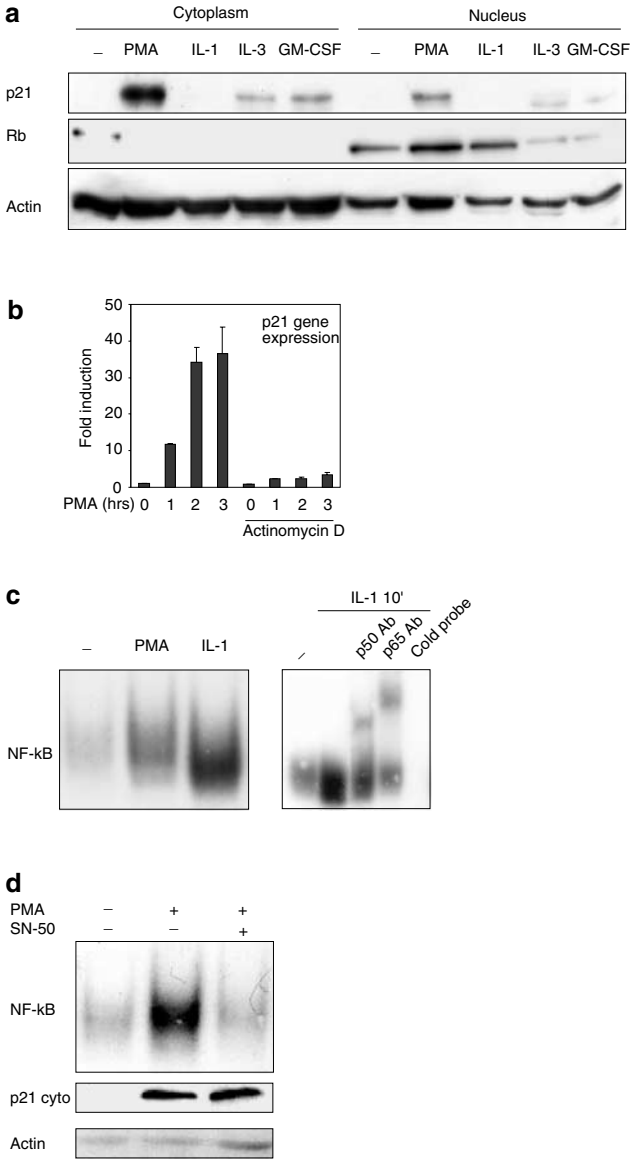

Figure 1 Expression and localization of p21 in monocytes. (a) Monocytes from healthy donors were isolated and cultured as described in Materials and methods, and stimulated with 50 nM PMA, 10 ng/ml IL-1β, 10 ng/ml IL-3 and 10 ng/ml GM-CSF for 24 h. Nuclear and cytoplasmic fractions were analysed by SDS-PAGE. Rb is used as a fractionation control. The data presented are representative for five individual experiments. (b) Monocytes (5×10^6) were cultured for 24 h in RPMI 1640 supplemented with 10% FCS, and subsequently stimulated for 0, 1, 2 or 3 h with 50 nM PMA, in the presence and absence of 1 μg/ml actinomycin D. RNA was isolated and cDNA was synthesized, as described in Materials and methods. This cDNA was subjected to real-time PCR, in order to analyse p21 gene expression, and was normalized against HPRT. The data presented are averaged from two individual experiments. (c) Monocytes were cultured for 6 h in the presence of 50 nM PMA or 10 ng/ml IL-1β. EMSAs were used to detect the DNA-binding activity of NF-κB in nuclear extracts. Controls with α-p50 and α-p65 antibodies for supershifts and cold probe competition are shown for IL-1-treated samples, in order to verify that the observed complex is specific and contains NF-κB. The data presented are representative for three individual experiments. (d) After culturing monocytes for 6 h in the presence of 50 nM PMA with or without pretreatment with the specific NF-κB inhibitor SN-50 (100 μg/ml), nuclear extracts were analysed for the DNA-binding capacity of NF-κB, in an EMSA. Cytoplasmic extracts were analysed for p21 content by SDS-PAGE. The data presented are representative of two individual experiments. Cyto = cytoplasmic.

localization. Correct fractionation is indicated by the absence of leakage of the nuclear Rb protein to the cytoplasm. Stimulation with IL-1β did not result in the appearance of p21 protein, despite the fact that IL-1 is capable of inducing NF-κB DNA-binding activity.[27–32] Since the appearance of p21 could reflect increased p21 gene expression or a diminished p21 protein degradation,[33] a real-time PCR was performed on PMA-stimulated monocytes. Treatment of monocytic cells from healthy donors with PMA upto 3 h clearly showed an upregulation of p21 gene expression, which could be effectively blocked by treatment with the polymerase II inhibitor actinomycin D (Figure 1b). This indicates that PMA can regulate p21 at the level of gene expression. Next, we determined whether NF-κB is activated by PMA and is required for PMA-induced p21 expression. Monocytes were stimulated with PMA and IL-1, and analysis of DNA binding by NF-κB was performed with an electrophoretic mobility shift assay (EMSA), as described in Materials and methods. PMA and IL-1 both induce NF-κB binding to its consensus binding sequence (Figure 1c). Supershift experiments with antibodies directed against the NF-κB subunits p50 and p65 resulted in complexes with a decreased mobility, indicating the presence of NF-κB in the observed complexes. Addition of a molar excess of unlabelled probe resulted in a complete disappearance of IL-1-induced DNA–protein complexes. In order to analyse if the PMA-induced NF-κB activation is involved in the upregulation of p21 expression, monocytes were stimulated with PMA in the absence and presence of the NF-κB inhibitor SN-50. As demonstrated in Figure 1d, SN-50 inhibited the induction of NF-κB DNA-binding activity

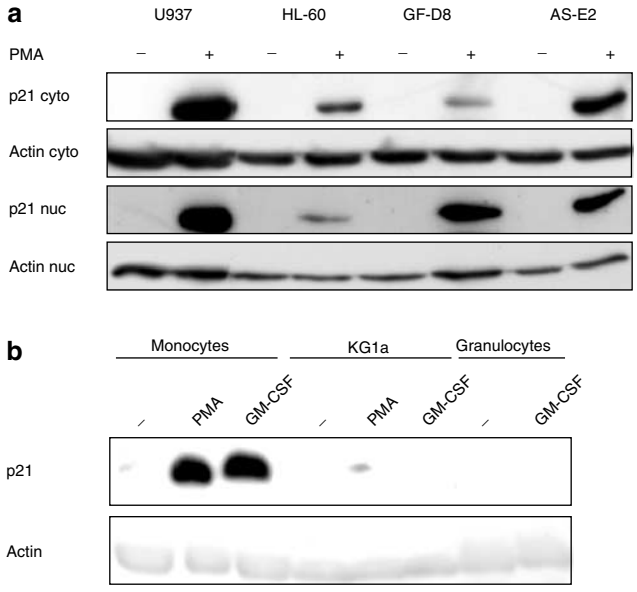

Figure 2 Expression and localization of p21 in haematopoietic cells. (a) Expression of p21 protein in U937, HL-60, GF-D8 and AS-E2 cells. After culturing these cells in the presence or absence of 50 nM PMA for 6 h, nuclear and cytoplasmic extracts were analysed for p21 levels by SDS-PAGE. The data presented are representative for two individual experiments. Cyto = cytoplasmic, nuc = nuclear. (b) Expression of p21 in the primitive cell line KG1a and granulocytes. Granulocytes were obtained from healthy donors and stimulated with 50 nM PMA or 10 ng/ml GM-CSF for 6 h. KG1a cells were stimulated with 50 nM PMA or 10 ng/ml GM-CSF for 6 h. Western blot analysis of whole extracts was performed to demonstrate p21 expression upon GM-CSF and PMA treatment. Monocytes stimulated with PMA and GM-CSF were used as positive controls. Actin was used as loading control. The data presented are representative of four individual experiments.

in response to PMA stimulation, but did not affect the PMA-induced p21 expression. These data indicate that in monocytes, NF-κB is not required for PMA-induced p21 expression.

To further investigate the expression and role of p21 in cells of monocytic and myeloid origin, granulocytes and the cell lines KG1a, HL-60, AS-E2, GF-D8 and U937 were stimulated with GM-CSF or PMA. In none of the unstimulated celllines, p21 could be detected in the nucleus or cytoplasm. Upon stimulation with PMA during 6 h, an increase in cytoplasmic and nuclear p21 was demonstrated in all cell lines studied, which was most prominent in the monocytic cell line U937 (Figure 2a). In the primitive KG1a cell line and in granulocytes, PMA- or GM-CSF-induced p21 expression was low or undetectable compared to monocytic cells (Figure 2b). These findings indicate that in haematopoietic cells, p21 expression is absent in unstimulated cells, and can be significantly upregulated after induction with PMA, GM-CSF or other cytokines, with the exception of the KG1a cells and granulocytes. In addition, while cells of the myeloid lineage showed comparable amounts of cytoplasmic and nuclear p21, monocytes were observed to have more p21 protein localized in the cytoplasm after PMA and GM-CSF treatment (Figures 1a, 2a).

Using chemical inhibitors, the role of various intracellular signalling routes in regulating p21 expression in monocytes was investigated. The inhibitors used were U0126, SB203580 and Ly294002, which block the MEK1, p38 kinase and the PI(3)K activity, respectively. In monocytes, neither U0126, SB203580, nor Ly294002 affected PMA- (Figure 3a) and GM-CSF (data not shown)-induced p21 expression, while the used inhibitors effectively interfered with the activation of downstream effectors (Figure 3b). The PKC inhibitors BIM and chelerythrine, however, strongly inhibited p21 expression. Low concentrations of BIM display activity on typical and atypical PKC isoforms.[34] Similar findings have been described for chelerythrine.[35] The PKC inhibitor calphostin-C, which has been described to act on classical and novel PKC isoforms,[35–37] had no effect on p21 expression (Figure 3a). In all cases, localization of the p21 protein was not affected by the use of different inhibitors. The absence of nuclear p21 after treatment with BIM and chelerythrin is probably due to the lowered expression levels, instead of relocalization. These findings indicate that in monocytic cells, a PKC-dependent pathway is responsible for the expression of the p21 protein, although it remains to be determined which PKC isoform is the main effector of this process.

p21 expression in AML

In haematopoietic cells, p21 is not expressed in unstimulated cells, but can be induced by PMA in the monocytic lineage in a PKC-dependent fashion. In order to see if a similar expression pattern exists in the malignant counterpart, p21 was studied in cryopreserved AML cells of either monocytic or myeloid origin. In contrast to normal unstimulated monocytes, a high constitutive expression of p21 was observed in 75% of the monocytic leukaemia (M4–5, $N=12$), with a predominant cytoplasmic localization (Figure 4a). In 50% of the myeloid leukaemias

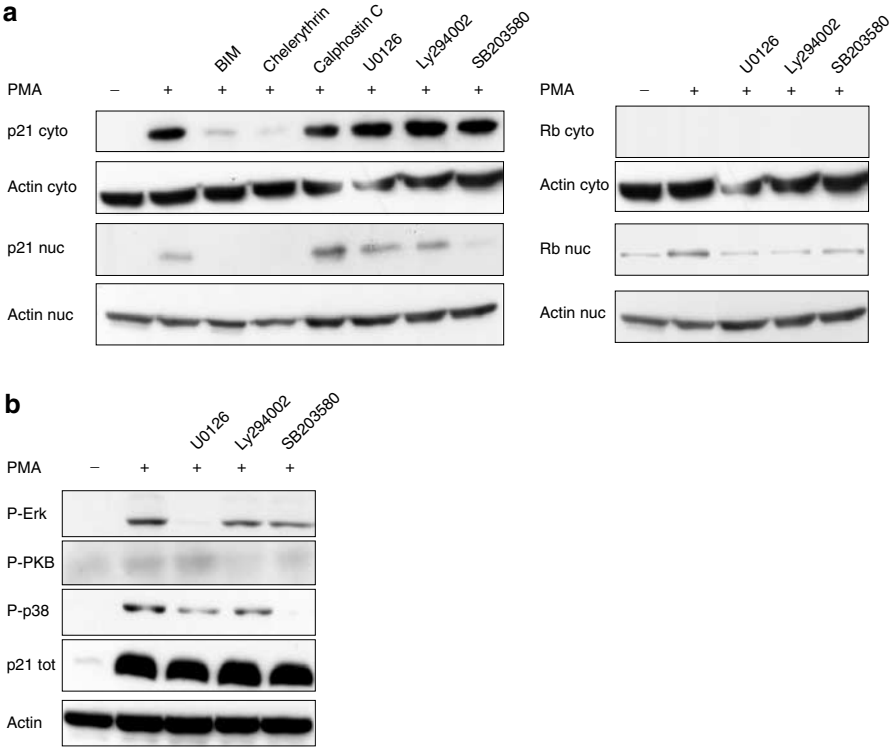

Figure 3 (a) Expression of p21 in monocytes is inhibited by the PKC inhibitors BIM and chelerythrin. Monocytes from healthy donors were isolated and cultured as described, and pretreated for 1 h with 1 μM BIM, 6 μM chelerythrin, 100 nM calphostin-C, 5 μM U0126, 5 μM Ly29002 and 1 μM SB203580, and stimulated with 50 nM PMA for 6 h. Nuclear and cytoplasmic fractions were analysed for p21 levels by SDS-PAGE. The data presented are representative for three individual experiments. Actin is used as a loading control, and proper fractionation is demonstrated by the lack of Rb immunoreactivity in the cytoplasmic fraction. Cyto = cytoplasmic, nuc = nuclear. (b) Validation of the effectiveness of the used inhibitors. Monocytes were pretreated with 5 μM U0126, 5 μM Ly29002 and 1 μM SB203580 for 1 h, and stimulated for 30 min with 50 nM PMA. P-Erk, P-PKB and P-p38 are shown. Actin is shown for loading control. The expression of p21 protein in the same samples was analysed after 6 h. Tot = total.

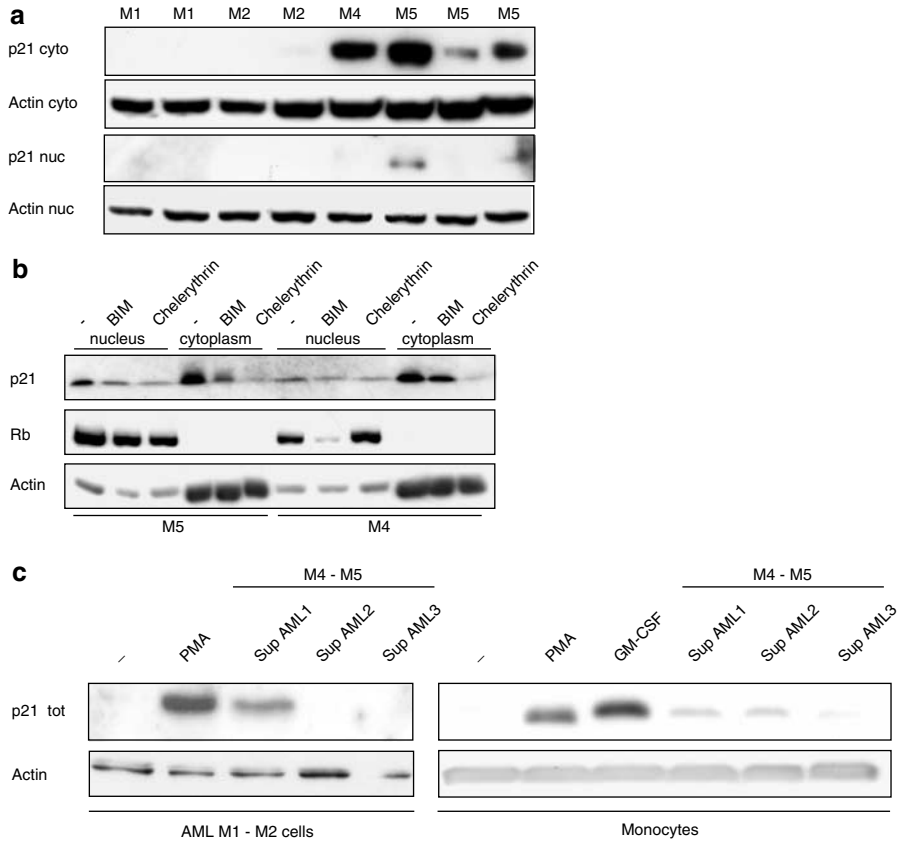

Figure 4 Expression of p21 in AML blasts. (a) AML blasts were isolated and cultured as described. Cytoplasmic and nuclear fractions were analysed for p21 content by SDS-PAGE. Eight representative cases are shown. The data presented are representative of two individual experiments. (b) AML blasts (M4–5) were isolated and cultured for 24 h in the presence of 1 μM BIM or 6 μM chelerythrin. The p21 protein was detected in cytoplasmic fractions by means of SDS-PAGE. As controls for subcellular fractionation, Rb protein was used. Two representative cases are shown. The data presented are representative of two individual experiments. (c) AML blasts (M1–2) were cultured for 24 h and stimulated with 50 nM PMA or conditioned AML medium from M4–5 cultures, to study the role of autocrine growth factors leading to p21 expression. Conditioned AML medium was obtained by culturing 3×10^6 \FS28 AML blasts (M4–5) for 3 days. The same conditioned media were added to normal monocytes, in order to analyse p21 expression. Stimulation with 50 nM PMA or 10 ng/ml GM-CSF was used as positive control. Actin is shown as loading control. The data presented are representative for two individual experiments. Cyto = cytoplasmic, nuc = nuclear.

(M1–2, $N=10$), p21 expression could not be detected without stimulation, or a low expression of p21 was observed, which was localized in both the nucleus and cytoplasm (Figure 4a; data not shown). These findings link aberrant p21 expression primarily to cells of monocytic origin. To demonstrate whether PKC is also involved in the spontaneous expression of p21 in AML cells, blast cells ($N=5$) with a high cytoplasmic p21 localization were exposed to the PKC inhibitors BIM and chelerythrin. Chelerythrin reduced p21 expression in all studied cases, while BIM inhibited the p21 expression in four of five studied cases (Figure 4b). The use of PKC inhibitors only affected the expression of p21 and not the localization, as demonstrated in Figure 4b. Rb was used as a fractionation control. These results underscore a role for PKC-dependent activation of p21 expression in malignant monocytic cells.

Next, we investigated whether the activated signal transduction pathway leading to constitutive p21 expression might be due to the autocrine production of a growth factor or a cell-autonomous effect. Therefore, after 3 days of culture, conditioned medium of monocytic AML blast (M4–5) cultures (supernatant) was added to cultures of myeloid AML blasts (M1-2), and p21 expression was analysed (Figure 4c). In one case, monocytic AML-conditioned medium induced p21 expression in myeloid AML blasts, while in the additional cases no effects were observed. Since these myeloid AML cells might have some inherent blockade, the conditioned medium was also added to monocytes of healthy donors. The AML-conditioned medium now induced p21 expression in normal monocytes, although not as high as PMA- or GM-CSF-induced p21 expression (Figure 4c). These results suggest that in monocytic leukaemia, the autocrine production of growth factors could lead to constitutive p21 expression. In addition, a cell-autonomous mechanism of p21 expression cannot be excluded, and might also contribute to this observation.

Cellular function of p21

It has been demonstrated that cytoplasmic p21 has antiapoptotic properties in response to stress, due to the interaction between p21 and ASK1.[10] To investigate whether this applies also to the effects of cytostatic agents, U937 cells overexpressing p21 and neomycin-transfected control cells were exposed to VP16 or H_2O_2. A significant lower percentage of apoptotic cells was observed in response to VP16 exposure in p21-overexpressing cells compared to control cells (20.0 ± 0.9 *vs* $55.8 \pm 3.8\%$, $N=8$, $P<0.01$) (Figure 5a). In addition, p21-negative ($N=6$) and p21-positive ($N=7$) AML blasts were subjected to VP16 treatment. As a measure of the apoptotic response, levels of activated caspase 3 were detected, as well as combined annexin

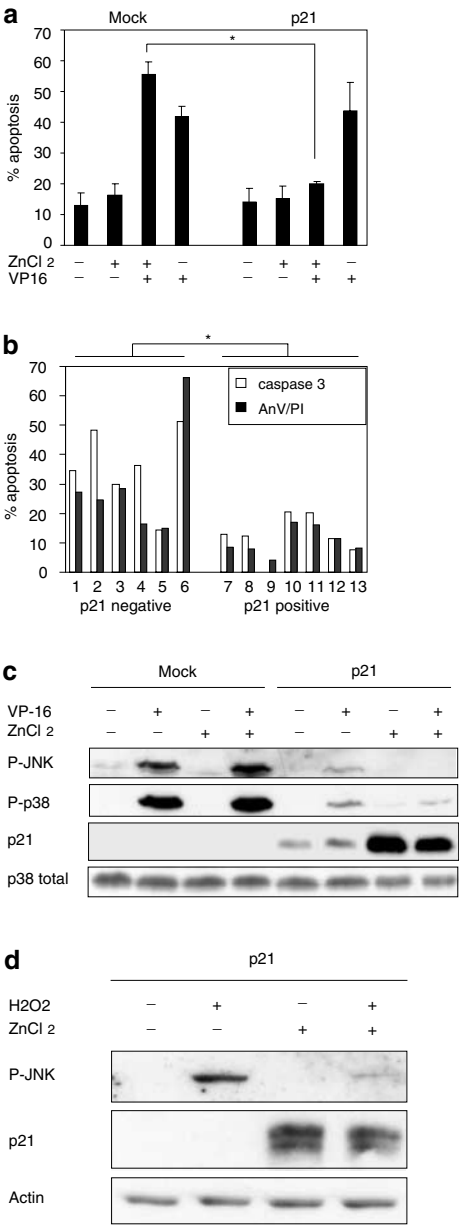

Figure 5 The p21 protein affects apoptosis induced by VP16 or H_2O_2. (a) Mock-transfected U937 cells or U937 cells stable transfected with a $ZnCl_2$-inducible p21 construct were cultured for 3 days in the presence of 180 μM $ZnCl_2$ to induce p21 expression. Next, the cells were exposed to 20 μg/ml VP16 for 24 h, and afterwards apoptosis was measured using the combined annexin V/PI staining procedure, as described in Materials and methods. The data presented are averaged from eight independent experiments, with error bars denoting standard errors. $^*P<0.01$. (b) AML blasts were isolated and cultured as described. After 24 h of exposure to 20 μg/ml VP16, apoptosis was measured using the combined annexin V/PI staining by flow cytometry. Also, levels of activated caspase 3 were measured using flow cytometry, as described in Materials and methods. For both experiments, $^*P<0.01$. (c) The mock- and stable-transfected U937 cells expressing p21 were cultured for 3 days with 180 μM $ZnCl_2$, and apoptosis was induced by culturing in the presence of 20 μg/ml VP16. Activation of JNK and p38 was studied in total cell extracts by Western analysis. The data presented are representative of five individual experiments. (d) Apoptosis was induced in U937 cells stably overexpressing p21, by culturing these cells for 24 h in the presence of 300 μM H_2O_2. Whole extracts were assayed for phosphorylated JNK by Western analysis. The data presented are representative of five individual experiments.

V/PI stainings, which were both measured using flow cytometry. The group of AML blasts with high cytoplasmic p21 expression ($N=7$) was significantly less sensitive to VP16-induced apoptosis as compared to AML blasts with low p21 expression levels ($N=6$), both for the active caspase 3 assay (respectively, 12.3 *vs* 42.3%, $P<0.01$) and the combined annexin V/PI experiments (respectively, 8.5 *vs* 25.8%, $P<0.01$) (Figure 5b). Next, it was investigated whether the reduced apoptosis corresponded to a reduced phosphorylation, and hence activation of the known downstream targets of ASK1, p38 and JNK, since these have been shown to be effectors of ASK1-mediated apoptosis.[38–40] As depicted in Figure 5c, the protective effect of p21 in the U937 cell line was associated with a reduced phosphorylation of p38 and JNK in p21-overexpressing cells in response to VP-16 triggering. Similar results were obtained with the stress response activator H_2O_2 (Figure 5d). To further underscore the connection between p21 and ASK1, coimmunoprecipitations were performed with p21 and ASK1 in two AML cases with high cytoplasmic p21 levels. For this coimmunoprecipitation, an anti-p21 antibody conjugated to agarose beads was used. As control for p21 expression and p21–ASK1 complex formation, untreated AS-E2 cells stimulated with 50 nM PMA for 16 h were used. As negative control, unstimulated AS-E2 cells were used, since unstimulated AS-E2 cells do not express p21 (Figure 2a). After 16 h of PMA (50 nM) stimulation, complex formation between p21 and ASK1 could be observed in the AS-E2 cell line, indicating that ASK1 co-precipitates with the p21 protein (Figure 6). In the AML blasts, ASK1 co-precipitated with p21 in all conditions shown. Although in the M5 AML cells, PMA treatment resulted in more ASK1 immunoreactivity, this is probably due to increased p21 expression in response to PMA. The complex formation between p21 and ASK1 itself, however, does not appear to be PMA dependent, since ASK1 can also be detected in p21 precipitates from untreated M5 AML cells. In short, we show that in monocytic leukaemias, cytoplasmic p21 functions as an antiapoptotic protein. This is demonstrated by a lowered sensitivity towards VP16-induced apoptosis, which is reflected by a decreased phosphorylation of the known downstream targets of ASK1, p38 and JNK. The observed complex formation between p21 and ASK1 further strengthens this hypothesis.

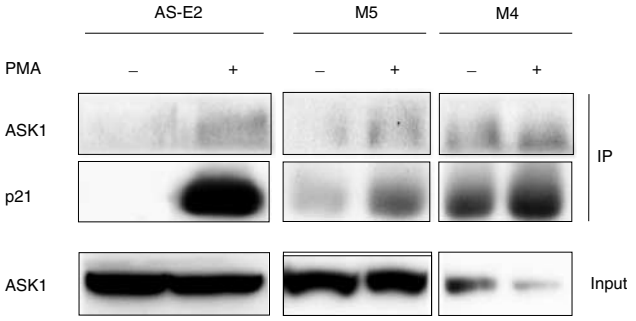

Figure 6 The p21 protein forms a complex with ASK1 in monocytic AML blasts and AS-E2 cells. AML blasts (10^7; M4–5) were cultured in the absence or presence of 50 nM PMA for 16 h as indicated. Cell lysates were subjected to p21 immunoprecipitation (IP), using an α-p21 antibody, conjugated to agarose beads. Immunoblotting was performed using α-p21 and α-ASK1 antibodies. Expression of ASK1 in all samples was confirmed by immunoblotting of total cell extracts; see the 'input' panel. As negative and positive controls, 10^7 AS-E2 cells were grown in the absence or presence of 50 nM PMA, respectively, and IP was performed. The data presented are representative of five individual experiments.

Discussion

The role of nuclear p21 as a cell cycle regulator by interaction with several cyclin-dependent kinases is well established.[12–16] Recent studies have indicated that p21 might have an additional function when the protein is localized in the cytoplasm. Cytoplasmic p21 is linked to the antiapoptotic process, by interacting with ASK1 or procaspase 3.[10,19,41,42] The present study indicates that in differentiating haematopoietic cells of monocytic and myeloid lineage, p21 can be selectively upregulated by a number of cytokines, which in monocytic cells is a PKC-dependent but NF-κB-independent process. A recent study in monocytes indicated a link between NF-κB activation and p21 upregulation, suggesting that p21 might accomplish the antiapoptotic effects of NF-κB.[9] However, in the present study, it is shown that NF-κB activity is not required for p21 expression. In monocytic leukaemia, constitutively expressed p21 is frequently observed in contrast to myeloid leukaemia, which is a PKC-dependent process. This is likely linked to the autocrine production of growth factors. However, it is not strictly excluded that a yet unknown upstream kinase or receptor mutation results in the constitutive activation of this signal transduction pathway, leading to constitutive p21 expression. The PKC-dependent expression of p21 is not linked to the MEK1 or PI(3)K signalling pathways. This is in contrast with other reports, where it was shown that p21 protein stability is enhanced by phosphorylation through PKB,[20] and where PMA-induced p21 expression is linked to nuclear distributed ERK.[43] In addition, phosphorylation of p21 on Thr^{145} is suggested to be a prerequisite for its cytoplasmic localization.[44] Whether the localization of p21 is dependent on the phosphorylation status of the protein in conjunction with the cellular setting is the subject of further research.

Finally, it is shown that p21 overexpression inhibits VP16-mediated apoptosis, as reflected by the inhibitory effects on the phosphorylation of p38 and JNK, known downstream targets of ASK1. In addition, p21 and ASK1 form a complex in cell lines and AML blasts, underscoring a strong connection between the antiapoptotic function of p21- and ASK1-mediated apoptosis. The results obtained in monocytes are in disagreement with the findings by Asada *et al*,[10] who demonstrated that unstimulated monocytes express p21. The cause of this difference is unclear, but might be related to the isolation procedure used.

The data presented here and other findings indicate that, in leukaemic cells, antiapoptotic proteins are regulated by different signalling routes, including the RAS/PI(3)K/NF-κB pathway,[4,6–8,45] the STAT3 and STAT5 pathways[5,46] and in monocytic blasts also at the level of p21 expression.[9,10,17] These findings illustrate that, until a single oncogenic defect that is responsible for the total spectrum of activation signals is defined, combination of inhibitors has to be used for chemotherapeutic interference with the distinct and overlapping activated signalling routes.

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